ATPase activity of the heat shock protein Hsp72 is dispensable for its effects on dephosphorylation of stress kinase JNK and on heat-induced apoptosis

Vladimir Volloch[a,b], Vladimir L. Gabai[a], Sophia Rits[a], Michael Y. Sherman[a,*]

[a] *Boston Biomedical Research Institute, 20 Staniford Street, Boston, MA 02114, USA*
[b] *Tufts University Biotechnology Center, 4 Colby St., Medford, MA 02155, USA*

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Abstract A major inducible heat shock protein, Hsp72, has previously been found to stimulate dephosphorylation (inactivation) of stress kinase JNK in heat-shocked cells and protect them from apoptosis. Using Rat-1 fibroblasts with constitutive expression of a human Hsp72 or its deletion mutant lacking an ATPase domain (C-terminal fragment (CTF)), we tested whether ATPase activity of Hsp72 is necessary for these effects. We found that expression of CTF markedly increased, similarly to the intact protein, JNK dephosphorylation in heat-shocked cells. As a result, JNK inactivation following heat shock occurred much faster in cells expressing either full-length or mutant Hsp72 than in parental cells and this was accompanied by suppression of heat-induced apoptosis. Thus, protein refolding activity of Hsp72 appears to be dispensable for its effect on JNK inactivation and apoptosis.

Key words: Heat shock protein; Hsp70 ATPase; JNK stress kinase; Apoptosis

1. Introduction

The major inducible heat shock protein, Hsp72, plays a key role in thermotolerance. Hsp72 expression was shown to increase the colony-forming ability following heat shock [1,2] as well as decrease heat shock-induced apoptosis [3–5] and necrosis [2]. Members of the Hsp70 family function as molecular chaperones in facilitating protein folding, translocation across membranes and degradation (see [6] for review). Therefore, Hsp72 was suggested to increase cell survival through binding to unfolded polypeptides and preventing protein aggregation as well as accelerating refolding or degradation of damaged proteins [7,8].

The chaperoning function of Hsp72 resulting in protein protection can be potentially important for prevention of heat-induced cell killing. The protein binding domain of Hsp70 was shown to be absolutely necessary for cell protection [9,10]. On the other hand, Li et al. demonstrated that the expression of a human Hsp72 deletion mutant (C-terminal fragment (CTF)) lacking the ATPase domain (and, consequently, the protein refolding activity) in Rat-1 cells was sufficient to protect cells from loss of the colony-forming ability after heat treatment [9]. Later, several studies have been done to assess the effect of expression of this Hsp72 mutant on some cellular parameters which are potentially related to cell survival. It was found that, similar to expression of the full-length Hsp72, expression of CTF reduced intranuclear protein aggregation in heat-shocked cells [10]. However, in contrast to full-length Hsp72, expression of CTF did not reduce inactivation of translation, nor did it accelerate recovery of transcription and translation following heat shock [11]. Furthermore, CTF expression, unlike expression of full-length Hsp72, did not accelerate inactivation of heat shock transcription factor HSF1 following recovery from heat shock [12]. Thus, it is obvious that some important activities of Hsp72 are lost in the mutant protein lacking the ATPase domain.

We and others have previously found that expression of Hsp72 suppressed heat-induced JNK activation [5,13,14]. Studying possible targets of Hsp72 in the JNK-signaling pathway, we found that heat shock and other protein-damaging stresses activate JNK via blockade of JNK dephosphorylation and that Hsp72 alleviates this JNK phosphatase inhibition [15]. Here, using a CTF mutant of Hsp72, we investigated whether ATPase activity of Hsp72 is necessary for the effect of Hsp72 on JNK dephosphorylation (inactivation). Since the effect of Hsp72 on JNK can also be critical for anti-apoptotic protection (see [16] for review), another question which we addressed was whether the ATPase domain is necessary for the anti-apoptotic effect of Hsp72 in heat-shocked cells.

2. Materials and methods

2.1. Cell lines

Parental Rat-1 fibroblasts and their variants expressing Hsp72 (MVH) and CTF (ΔBgl) were kindly provided by Dr G.C. Li. Cells were grown in DMEM supplemented with 10% fetal bovine serum and used for experiments at 40–70% confluency.

2.2. Measurement of JNK activity

JNK activity was measured as described previously [15]. Briefly, cells were lysed in a buffer containing 40 mM HEPES, pH 7.5, 50 mM KCl, 1% Triton X-100, 2 mM DTT, 1 mM Na_3VO_4, 50 mM β-glycerophosphate, 50 mM NaF, 5 mM EDTA, 5 mM EGTA, 1 mM PMSF, 1 mM benzamidine, 5 μg/ml of each leupeptin, pepstatin A, aprotinin. The lysates were clarified by centrifugation in a microcentrifuge at 15 000 rpm for 5 min. The protein concentration was measured in the supernatants after which they were diluted with the lysis buffer to achieve equivalent concentrations in all samples. Then, samples were subjected to polyacrylamide gel electrophoresis and Western immunoblotting using JNK antibody specific to the activated (phosphorylated) form of JNK (Promega, WI, USA), which allowed us to measure activity of two major isoforms (46 kDa JNK1 and 54 kDa JNK2). Secondary antibodies conjugated with peroxidase were visualized with ECL substrates (Amersham, Arlington Heights, IL, USA) and resulting films were quantified by densitometry.

2.3. Measurement of JNK phosphatase activity

In vivo JNK phosphatase activity was measured as described earlier

*Corresponding author. Fax: (1) (617) 912-0308.
E-mail: sherman@alpha.bbri.org

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[15]. Cells were exposed to heat shock and transferred back to 37°C. Then, further JNK phosphorylation (activation) was completely inhibited by addition 10 mM 2-deoxyglucose and 5 μM rotenone (a mitochondrial inhibitor), which rapidly deplete ATP, and cell samples were taken at different time points. The rate of JNK dephosphorylation was assessed under these conditions by immunoblotting with the antibody to activated JNK as above.

2.4. Apoptosis quantification

The level of apoptosis was measured by fluorescent microscopy using Hoechst-33342 DNA-specific dye (10 μM). Shrunken cells with condensed or fragmented nuclei were counted as apoptotic.

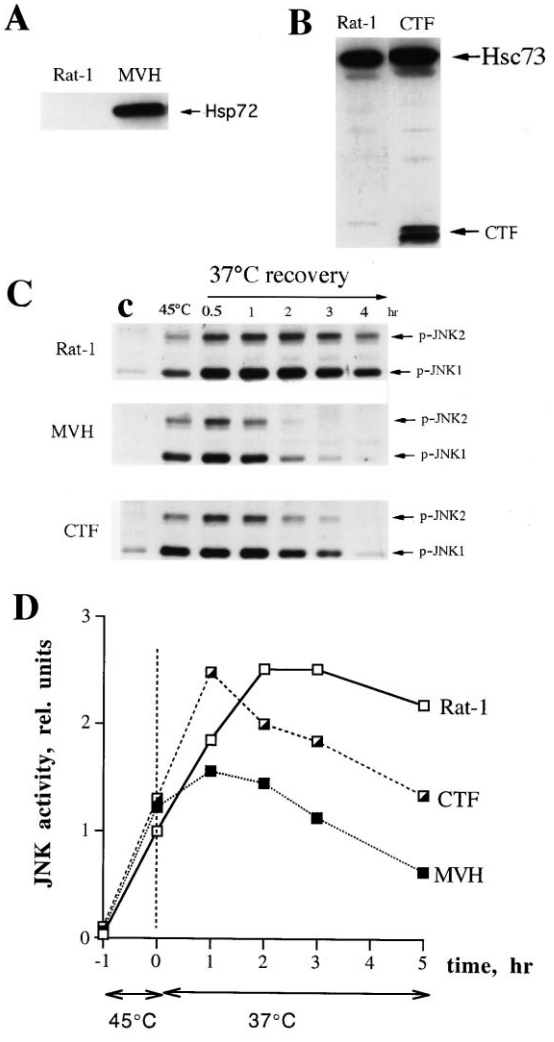

Fig. 1. CTF expression accelerates JNK inactivation following heat shock. (A) Hsp72 expression in MVH cells (as measured by immunoblot with SPA810 monoclonal antibody (MAb) to Hsp72). (B) CTF expression in Rat-1 cell variant (as measured by immunoblot with SPA820 MAb to Hsp72/Hsc73). (C and D) JNK activity in control (Rat-1), Hsp72 (MVH) and CTF-expressing cells after heat shock. Rat-1 cells and their variants were subjected to heat shock (45°C, 30 min, C or 50 min, D), then transferred to the normal temperature and JNK activity was measured by antibodies to phosphorylated (active) JNK at the time points indicated. In D, JNK1 activity immediately after heat shock in Rat-1 cells was taken as one relative unit.

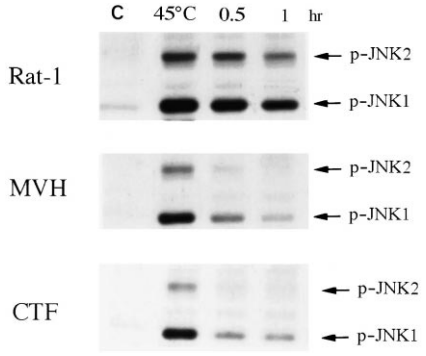

Fig. 2. CTF expression increases JNK phosphatase activity after heat shock. Rat-1, MVH (Hsp72) or CTF-expressing cells were subjected to heat shock (45°C, 30 min) and, following 2 h of recovery at 37°C, JNK dephosphorylation (JNK phosphatase activity) was measured under ATP-depleting conditions at the indicated time points. Under these conditions, JNK phosphorylation by upstream kinases was totally suppressed. Therefore, the rate of JNK dephosphorylation reflects JNK phosphatase activity (see Section 2 and [15] for further details).

3. Results

3.1. Expression of the ATPase-deficient mutant of Hsp72 (CTF) accelerates JNK inactivation in heat-shocked cells

To elucidate the effect of deletion of the ATPase domain of Hsp72 on activation of stress kinase JNK, we first used Rat-1 fibroblasts that constitutively express full-length Hsp72 (see Section 1). In contrast to PEER lymphoid cells and IMR90 fibroblasts [5,13,14], expression of Hsp72 in Rat-1 cells did not suppress initial JNK activation following heat shock (45°C, 30 or 50 min) but strongly accelerated the decrease in JNK activity (Fig. 1). With CTF-expressing cells, similarly, initial JNK activation following heat shock was not suppressed, but JNK inactivation was greatly accelerated (Fig. 1). In fact, the level of JNK activity fell to 50% of maximum 5 h after heat shock (45°C, 50 min) in CTF-expressing cells versus projected 14 h in parental cells (Fig. 1D).

3.2. CTF accelerates JNK dephosphorylation in heat-shocked cells

The above data show that expressions of Hsp72 and its mutant lacking the ATPase domain exert similar effects on JNK activity in Rat-1 cells. Since the effect of Hsp72 on JNK is associated with alleviation of inhibition of JNK dephosphorylation (phosphatase) [15], we studied how CTF expression affects JNK phosphatase. To assess the activity of JNK phosphatase(s), we measured the rate of JNK dephosphorylation under the conditions when upstream JNK-activating kinases are completely suppressed [15]. We found that, as expected, in parental Rat-1 cells following heat shock, JNK phosphatase(s) activity was very low (Fig. 2). However, in the heat-stressed cells that express Hsp72, the activity of JNK phosphatase(s) was much higher than that of the parental heat-stressed cells and so it was in cells expressing CTF (Fig. 2). Thus, CTF, like Hsp72, accelerates JNK inactivation by increasing the JNK dephosphorylation in heat-shocked cells.

3.3. CTF expression protects cells from heat-induced apoptosis

Hsp72-mediated suppression of JNK may be critical for the

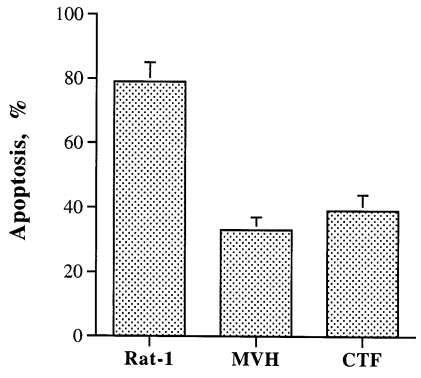

Fig. 3. CTF expression protects cells against heat-induced apoptosis. Rat-1, MVH (Hsp72) and CTF-expressing cells were subjected to heat shock (45°C, 50 min) and their apoptosis (mean ± S.D.) was determined 24 h later by Hoechst-33342 staining.

anti-apoptotic effect of this protein, although some other targets may also exist (see [16–18] for review). Because of a close similarity between effects of Hsp72 and CTF on JNK, we suggested that protein refolding activity of Hsp72 may also be unnecessary for its anti-apoptotic effect. To test this possibility, Rat-1 cells were subjected to severe heat shock (45°C, 50 min) which caused prolonged JNK activation (see Fig. 1D). This stress caused apoptosis in nearly 80% of parental cells within 24 h as assessed by chromatin condensation (Fig. 3) and by cleavage of a substrate of caspases, poly-(ADP ribose) polymerase (not shown). Hsp72 expression, as expected, decreased cell death about 3-fold and a significant (about 2-fold) decline in the number of apoptotic cells was observed in the CTF-expressing cell line (Fig. 3). Therefore, the protein refolding activity of Hsp72 is indeed dispensable for its anti-apoptotic effect.

4. Discussion

CTF of Hsp72 contains the peptide binding domain but lacks ATPase activity of the intact molecule. Accordingly, it can bind to damaged proteins but cannot dissociate from them and therefore cannot refold them. However, like constitutive expression of Hsp72, expression of CTF (called ΔBgl in [9]) was shown to protect Rat-1 cells from heat shock, as measured by the preserving of the cell's colony-forming ability, and such a protection is reminiscent of the protective effect of Hsp72 [9]. This similarity raised the possibility that CTF, like Hsp72, can also protect cells from heat-induced apoptosis and activate JNK dephosphorylation in heat-shocked cells [5,15]. Here, we tested this possibility and found that the potential of Hsp72 to accelerate JNK inactivation and prevent apoptosis is attributed to its peptide binding domain, whereas protein refolding activity of Hsp72 appears to be irrelevant to these effects.

The effect of CTF on the JNK phosphatase may provide an important clue to the mechanism of regulation of a JNK phosphatase by intact Hsp72. One of the possibilities may be that the JNK phosphatase is directly damaged by heating and that Hsp72, acting as a molecular chaperone, refolds the phosphatase and restores its activity. However, this interpretation is inconsistent with our observation that CTF facilitates JNK dephosphorylation and JNK inactivation, since CTF cannot refold damaged proteins due to the lack of ATPase activity. Therefore, we propose another mechanism by which Hsp72 regulates a JNK phosphatase. According to this mechanism, damaged proteins accumulated in heat-shocked cells bind to Hsp72, thus sequestering it. This shifts the equilibrium leading to dissociation of Hsp72 from JNK phosphatase and, consequently, to inactivation of the phosphatase. On the other hand, an increase in the level of Hsp72 would preserve the equilibrium, favoring association of Hsp72 with the phosphatase and consequently, JNK phosphatase activity will be maintained at a higher level even under heat shock conditions. In such a model, CTF would act similarly to Hsp72, except that it would associate with JNK phosphatase constantly, thus leading to its permanent activation.

The above mechanism of regulation of JNK phosphatase by Hsp72 predicts that Hsp72 would be able to suppress JNK activation even in response to stresses which do not cause protein damage. Indeed, suppression of JNK activation in Hsp72-expressing fibroblasts upon IL-1 and UV treatments was observed earlier [19]. Moreover, activation of JNK through the expression of upstream components of the JNK-signaling cascade, MEKK1 and Cdc42, was also suppressed by Hsp72 as well as by CTF mutant [19]. We suggest, therefore, that, besides its well-known function in protein refolding, Hsp72 can modulate JNK phosphatase activity.

Another novel important finding of this study is that ATPase activity of Hsp72 is dispensable for the anti-apoptotic effect of this protein (Fig. 3). The question arises whether the effect of CTF on JNK phosphatase may account for its anti-apoptotic effect. Although in Rat-1 cells, CTF as well as Hsp72 expression did not suppress initial JNK activation following heat shock, it did accelerate JNK inactivation during recovery (Fig. 1C,D). In a separate work, we demonstrated that it is the duration of JNK activation following heat shock rather than the initial level of JNK activity that is relevant to induction of apoptosis, at least in Rat-1 cells (Volloch et al., submitted). Therefore, it appears that the effect of CTF on JNK activity is also responsible for the anti-apoptotic effect of this protein.

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